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Award Number: DAMD17-99-1-9273

TITLE: The Regulatory Interactions of p21 and PCNA in Human  
Breast Cancer

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REPORT DATE: July 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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# REPORT DOCUMENTATION PAGE

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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 2000		3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jul 99 - 30 Jun 00)	
4. TITLE AND SUBTITLE The Regulatory Interactions of p21 and PCNA in Human Breast Cancer				5. FUNDING NUMBERS DAMD17-99-1-9273	
6. AUTHOR(S) Derek Hoelz Linda Malkas, Ph.D.					
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Maryland School of Medicine Baltimore, Maryland 21201 E-MAIL: dhoelz@som.umaryland.edu				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES					
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited					12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words)  To better understand the role of DNA replication in breast cancer, it is essential to examine the machinery that carries out the DNA synthetic process. Our laboratory has successfully purified a complex of proteins from breast cells that is fully competent to carry out T-antigen dependent, SV40 origin specific DNA replication <i>in vitro</i> , which we have termed the DNA synthesome. Analysis of the constituent proteins of the DNA synthesome of malignant and non-malignant breast cells by two-dimensional polyacrylamide gel electrophoresis (2D PAGE) has uncovered a modification in an essential DNA replication protein, proliferating cell nuclear antigen (PCNA). 2D PAGE analysis revealed that PCNA of malignant breast cells resolves as two distinct species, an unmodified form and a modified form, while PCNA present in non-malignant cell resolves exclusively as one form, the modified form. PCNA functions by forming a trimeric "sliding clamp" that encircles the DNA and interacts with the DNA polymerases $\delta$ and $\epsilon$ . Polymerase $\delta$ carries out leading strand DNA synthesis, and although a role for polymerase $\epsilon$ has not yet been ascribed, it has been hypothesized to function in DNA repair. Another protein that interacts with PCNA is p21 <sup>WAF1/CIP1/SDI1</sup> . P21 is a CDK inhibitor that, when induced by p53 in response to DNA damage, binds PCNA and effectively competes away polymerase $\delta$ leading to the efficient inhibition of DNA replication. This inhibition impedes the replication of damaged DNA and theoretically allots time for the cell to repair its damaged DNA. Therefore, any alterations of the PCNA molecule could potentially abrogate p21 binding leading to replication of damaged DNA and/or insufficient time for DNA repair. It is our goal to study the interaction of p21 with the modified and non-modified forms of PCNA and to investigate any functional consequences alterations in PCNA/p21 binding may have on DNA replication, DNA repair, and DNA replication fidelity.					
14. SUBJECT TERMS Breast Cancer, MCF7, p21, synthesome, DNA replication, DNA fidelity, cell proliferation					15. NUMBER OF PAGES 17
					16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified		18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified		19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	
					20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)  
Prescribed by ANSI Std. Z39-18  
298-102

20010228 102

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## **INTRODUCTION**

To better understand the role of DNA replication in breast cancer, it is essential to examine the machinery that carries out the DNA synthetic process. Our laboratory has successfully purified a complex of proteins from breast cells that is fully competent to carry out T-antigen dependent, SV40 origin specific DNA replication *in vitro* (Li *et al.*, 1984) which we have termed the DNA synthesome (Malkas *et al.*, 1990; Coll *et al.*, 1997). Analysis of the constituent proteins of the DNA synthesome of malignant and non-malignant breast cells by two-dimensional polyacrylamide gel electrophoresis (2D PAGE) has uncovered a modification in an essential DNA replication protein, proliferating cell nuclear antigen (PCNA) (Bechtel *et al.*, 1998). 2D PAGE analysis revealed that PCNA of malignant breast cells resolves as two distinct species, a unmodified form and a modified form, while PCNA present in non-malignant cells resolves exclusively as one form, the modified form. PCNA functions by forming a trimeric “sliding clamp” that encircles the DNA and interacts with the DNA polymerases  $\delta$  and  $\epsilon$  (Krishna *et al.*, 1994; Zhang *et al.*, 1998; ). Polymerase  $\delta$  carries out leading strand DNA synthesis, and although a role for polymerase  $\epsilon$  has not yet been ascribed, it has been hypothesized to function in DNA repair (Hindges *et al.*, 1997). Another protein that interacts with PCNA is p21<sup>WAF1/CIP1/SDI1</sup> (Li *et al.*, 1994; Luo *et al.*, 1995). P21 is a CDK inhibitor that, when induced by p53 in response to DNA damage, binds PCNA and effectively competes away polymerase  $\delta$  leading to the efficient inhibition of DNA replication (Waga *et al.*, 1994; Podust *et al.*, 1995; Warbrick *et al.*, 1997). This inhibition impedes the replication of damaged DNA and theoretically allots time for the cell to repair its damaged DNA. Therefore, any alterations of the PCNA molecule could potentially abrogate p21 binding leading to replication of damaged DNA and/or insufficient time for DNA repair. It is our goal to study the interaction of p21 with the unmodified and modified forms of PCNA and to investigate any functional consequences alterations in PCNA/p21 binding may have on DNA replication, DNA repair, and DNA replication fidelity.

## **Progress Report**

### **Purification and separation of the two forms of PCNA**

Two dimensional polyacrylamide gel electrophoresis (2D PAGE) and electroelution of the different forms of PCNA in MCF7 cells was performed in an attempt to purify the two forms of PCNA. Initially, MCF7 cells were fractionated to a clarified nuclear extract and post-microsomal (NE/S3) fraction and placed onto a Phosphocellulose column. Phosphocellulose dissociated the synthesize multiprotein complex and resolves PCNA from the DNA polymerases and other synthesize associated DNA replication proteins. The column eluate fraction containing PCNA was then passed over a hydrophobic Phenyl Sepharose column followed by anion exchange chromatography (Q Sepharose). Purified PCNA was then loaded onto 2D PAGE and resolved. One of the gels was silver stained and analyzed using the Melanie II software. Analysis predicted the general location of the PCNA species and empirically predicted the isoelectric points (pIs) of the two species. The pIs for PCNA were 4.74 for the unmodified (cancer specific) form, which is close to the theoretical pI for PCNA (4.56), and 6.96 for the modified (non-malignant) form. The unmodified form and modified form of PCNA were then excised from four parallel 2D PAGE gels and electroeluted into 25mM HEPES buffer pH 7.2 using a Mini Whole-Gel Eluter (Bio-Rad). The presence of PCNA in the electroeluted fractions was determined by slot blotting (Life Technologies, Inc.), and the fractions containing PCNA were subsequently tested by 2D PAGE. The 2D gels confirmed the presence of the unmodified form in the electroelute; however, 2D analysis of the modified form of PCNA in the electroelute demonstrated the exclusive presence of the cancer-specific form. It was concluded from these data that the modification of PCNA was lost in either the electroelution or, more probably, the second dimension SDS PAGE step. Because of the apparent loss of modification, new approaches to separating the two forms of PCNA have begun to be developed.

In order to separate the two forms of PCNA, an alternative chromatographic protocol will be employed. The Q-Sepharose chromatography has been replaced with cation exchange chromatography (SP Sepharose). PCNA eluted off the Phenyl Sepharose column will be dialyzed into 50mM potassium phosphate buffer with a pH of 3.0. Because the pH of the buffer is below the pIs for the two forms of PCNA, the molecules will have a positive character and bind to the SP Sepharose. The unmodified form can now be eluted from the column in 50mM potassium phosphate pH 5.75, and because the pH of this buffer is still below the pI of the modified form, it will still bind the column and not be eluted. The modified form can then be eluted into 50mM potassium phosphate pH 8.0. This will enable us to better study the unmodified and modified forms of PCNA and their interactions with p21.

### **Cloning PCNA into a protein expression vector**

In order to study PCNA and its modification(s) we are going to express and modify the protein *in vitro*. To do this we cloned the PCNA from MCF7 cells. The cDNA sequence encoding the entire PCNA translation unit was prepared using Superscript Preamplification System First Strand DNA Synthesis (Life Technologies, Inc.) followed by cloning of the amplified cDNA into the vector pCR2.1 (Invitrogen) according to the manufacturers instructions. Total RNA was isolated using Trizol reagent (Life Technologies, Inc.). Second strand DNA synthesis was carried

out by priming the first strand cDNA with oligonucleotide 5'-GCGTTGTTGCCACTCCGC-3' on the 5' end of the cDNA and 5'-GCAGTTCTCAAAGAGCTTAG-3' on the 3' end of the cDNA and amplifying the primed first strand using reverse transcriptase PCR.

Subcloning of the cDNA was done using the pDUAL expression and cloning kit according to the manufacturer's protocol (Stratagene). Briefly, Eam1104I restriction sites were engineered onto the 5' ends of the PCR primers 5'-ATGTTTCGAGGCGCGCCTGGTCCAG-3' and 5'-AGATCCTTCATCCTCGATCTTGGGAGC-3' and the amplified PCNA cDNA was inserted into the pDUAL expression vector. Purification of PCNA is accomplished using Calmodulin Affinity Resin (Stratagene) which specifically binds a Calmodulin Binding Protein (CBP) tag fused to the C-terminus of the protein.

## **2D PAGE, tryptic digestion of PCNA, and MALDI-TOF mass spectrometry**

A new approach has been employed to identify the amino acid sequence harboring the PCNA modification. MCF7 cells were fractionated and PCNA was purified through a Q Sepharose chromatography as outlined in above. The two forms of PCNA were then resolved on 2D PAGE and visualized by silver staining using Silver Stain Plus (Bio-Rad). The spots corresponding to PCNA are then excised from the gel and digested with trypsin. The resultant peptide fragments are then resolved using an MALDI-TOF mass spectrometer in the negative ion, reflectron mode. The molecular masses of the peptide fragments obtained by MALDI-TOF mass spectrometry are then used to search protein sequence databases available on the inter-net. Sites of post-translational modification can then be determined by increases in the apparent molecular masses of the peptide fragment(s). The mass difference of these shifted peptide fragments will give insight into the identity and location of the modification(s) on PCNA.

Using a Voyager DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, Inc.) we were able to resolve peptide fragments derived from PCNA excised from 2D PAGE, and by searching the proteomics database Protein Prospector (UCSF), we were able to identify the protein as PCNA by its tryptic digest pattern. Further work will enable us to resolve the modified fragment(s) identifying the location and nature of the modification(s).

## ***In vitro* transcription and translation of GST-p21**

To study the interactions of p21 with the two forms of PCNA, GST-p21 protein was produced *in vitro* using *E. coli* T7 S30 Extract System for Circular DNA (Promega) for use in Far Western blotting experiments (see below). GST-p21 was labeled by addition of <sup>3</sup>H-leucine to the *in vitro* transcription and translation reaction and purified on a Glutathione Sepharose column (Amersham Pharmacia). Presence of *in vitro* protein product was confirmed by SDS-PAGE and autoradiography.

## **Co-immune precipitation, GST pull-down assays, and Far Western blotting**

To demonstrate that the modification(s) on PCNA effects its ability to interact with p21, three different experimental approaches were taken. First, the interaction of p21 with the two forms of PCNA was examined by co-immune precipitation. MCF7 cells were fractionated to a NE/S3 and

incubated with monoclonal anti-p21 antibody (DF10, Oncogene Research) at 4°C for 2 hours. The antibodies were then bound to Protein-A-Agarose (Oncogene Research) and washed. The precipitate was then resolved by 2D PAGE, transferred to nitrocellulose, and Western blotted using anti-PCNA antibody (PC10, Oncogene Research) (Figure 4). Secondly, GST pull-down experiments were performed. GST-p21 was purified from inclusion bodies *E. coli* BL21 (DE-3) cells (Stratagene) and purified using Glutathione Sepharose. MCF7 NE/S3 was then added to the GST-p21 conjugated Glutathione Sepharose beads and incubated at 4°C for 2h. The beads were washed and loaded onto 2D PAGE. The 2D gels were transferred to nitrocellulose and Western blotted with PCNA antibody. The co-immune precipitation and GST pull-down experiments showed similar results. They both demonstrated the presence of PCNA; however, the precipitated PCNA has a pI in-between that of the two forms of PCNA found in the MCF7 NE/S3 control. Another assay used to elucidate binding of p21 to PCNA was Far Western blotting. Far Western blotting utilizes a Western blotting approach that uses a labeled p21 instead of PCNA antibody to detect PCNA. MCF7 NE/S3 was first resolved on 2D PAGE and transferred to PVDF membrane. The proteins immobilized on the membrane were then denatured in buffer containing 6M guanidine HCl for 1h, slowly refolded in 3M guanidine HCl and step-wise down to 0.187 M and finally into buffer. The re-natured membranes were incubated with labeled GST-p21 (see above) over night at 4°C. The membranes were washed three times with buffer, dried, sprayed with En<sup>3</sup>Hance (New England Nuclear) and exposed to autoradiography at -80°C. Despite numerous attempts, we were unable to detect p21 binding to PCNA on 2D gels by Far Western blotting. Although spots are visible on the autoradiographs, indicating p21 binding, none of the spots overlap PCNA visualized by Western blotting of 2D gels run in parallel. Due to the inability of these experiments to demonstrate that the modification(s) on PCNA effects p21 binding, we have begun to take a new experimental direction.

### **New directions for analysis of the interaction between p21 and the two forms of PCNA**

To definitively show that the modification(s) on PCNA effects its ability to interact with p21, we have now begun to use BIAcore 2000 surface plasmon resonance (SPR) mass spectrometer (BIAcore, Inc). Briefly, the unmodified form and modified form of PCNA will be bound to BIAcore CM-5 chips by carbamidate linkage. Next, varying concentrations of p21 will then be passed over the chip surface and binding events will be detected by changes in molecular mass. The data collected for the binding of p21 to the unmodified and modified forms of PCNA will then be converted into dissociation constants ( $K_d$ ) using BIAevaluation software version 2.1 (BIAcore, Inc.) and Scatchard analysis. In addition, it has also come to our attention that the bacterially produced p21, which has been utilized in previously published BIAcore experiments, would not be optimal for  $K_d$  determination due the inevitable mis-folding of a percentage of p21 during the inclusion body purification. To address this issue we will use insect *sf9* cells to produce our recombinant p21. The p21 cDNA has been amplified using the PCR primers 5'-ATGTCAGAACCGGCTGG-3' with a 5' BamHI site and 5'-GGGCTTCCTCTTGGAGA-3' with a 5' EcoRI site and a 6X HIS tag. The amplified fragment was then sub-cloned into pFastBac1 expression vector using the Bac-TO-Bac Baculovirus Expression System (Life Technologies, Inc.), p21 will be stably transfected into the *sf9* cells, which will allow us to generate a purified, properly folded recombinant protein leading to a more reliable  $K_d$  value.

## **Key Research Accomplishments**

- **Performed 2D PAGE and electroeluted PCNA**
- **Tryptic digested PCNA excised from 2D PAGE and analyzed peptide fragments by MALDI-TOF mass spectrometry**
- **Cloned PCNA into pDUAL plasmid expression vector**
- **Co-immune experiments using anti-p21 antibody and analysis of precipitated PCNA by 2D PAGE**
- **Precipitation of PCNA by GST-p21 and analysis by 2D PAGE**
- ***In vitro* transcription, translation and purification of GST-p21**
- **Far Western blotting of 2D PAGE with <sup>3</sup>H labeled GST-p21**

## **Reportable Outcomes**

### **Abstracts**

Hoelz, D.J., Park, M., Dogruel, D., Bechtel, P., Sekowski, J., Xiang, H.Y., Hickey, R.J., Malkas, L.H. (2000): Analysis of a Malignant Cell's DNA Replication Apparatus by Mass Spectrometry. Scientific Proceeding of the 91<sup>st</sup> Annual Meeting of the American Association for Cancer Research. 41: 847.

### **Papers**

Hoelz, D.J., Bechtel, Hickey, R.J., Malkas, L.H. (2000): Purification of the Malignant Form of Proliferating Cell Nuclear Antigen from Breast Cancer cells. Manuscript in preparation.

## **Conclusions**

- **The unmodified form of PCNA has a pI of 6.96**
- **The modified form of PCNA has a pI of 4.76**
- **The modification to PCNA is apparently lost upon 2D PAGE and its subsequent electroelution from the gel**
- **The two forms of PCNA can be purified by ion-exchange chromatography taking into account their pIs**
- **Co-immune precipitation of PCNA with anti-p21 antibody produces a species of PCNA with an apparent pI different than that of the unmodified and modified forms**
- **GST-p21 pull down assays also produce a species of PCNA with a different pI identical to that of the co-immune precipitation**
- **Labeled recombinant GST-p21 is unable to bind PCNA in the Far Western experiments, which could be a function of the loss of modification mentioned above**

## **REFERENCES**

- Acharya, S., Wilson, T., Gradia, S., Kane, M.F., Guerrette, S., Marsischky, G., Kolodner, R., and Fishel, R. (1996):hMSH2 forms specific mispair binding complexes with hMSH3 and hMSH6. P.N.A.S. 93:13629-13634.
- Applegren, N., Hickey, RJ, Kleinschmidt, A.M., Zhou, Q., Wills, P., Coll, J., Bachur, N., Swaby, R., Wei, Y., Quan, J.Y.,Lee,M.Y., and Malkas, L.H. (1995): Further Characterization of the Human Cell Multiprotein DNA Replication Complex. J. of Cell. Biochem. 54 :32-46.
- Boulikas, Teni.(1991) Relation between carcinogenesis, chromatin structure and poly(ADP) ribosylation. Anticancer Res.11:489-528.
- Brenot-Bose, F., Gupta, S., Margolis, R.L., Fotedar, R. Changes in the subcellular localization of replication initiation proteins and cell cycle proteins during G<sub>1</sub>-to S-phase transition in mammalian cells. Chromosoma 103:517-527.
- Brugarolas, J., Chandrasekara, C., Goron, J., Beach, D., Jacks, T., Hannon, G. (1995) Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature 377:552-557.
- Chen, I-T., Smith, M., O'Connor, P., Fornace, A. (1995) Direct interaction of GADD45 with PCNA and evidence for competitive interaction of GADD45 and p21Waf1/Cip1 with PCNA. Oncogene 11(12):1931-1937.
- Chen, I-T. Akamatsu, M., Smith, M., Lung, F-D., Duba, D., Roller, P.P., Fornace, A.J., O'Connor, P. (1995) Characterization of p21Cip1/Waf1 peptide domains required for cyclin E/Cdk2 and PCNA interaction. Oncogene 12(3):595-607.
- Chen, J., Jackson, P., Kischer, M., Dutta, A. (1995) Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. Nature 374:386-388.
- Chen, J., Chen, S., Saha, P., Dutta, A. (1996) p21Cip1/Waf1 disrupts the recruitment of human Fen1 by proliferating -cell nuclear antigen into the replication complex. P.N.A.S. 93:11597-11602.
- Chen, J., Peters, R., Saha, P., Lee, P., Theodoras, A., Pagano, M., Wagner, G., Dutta, A. (1996) A 39 amino acid fragment of the cellcycle regulator p21 is sufficient to bind PCNA and partially inhibit DNA replication in vivo. Nuc. Acids Res. 24(9):1727-1733.
- Chen, Y.Q., Ciprano, S.C., Arenkiel, J.M., Miller, F.R. (1995) Tumor suppression by p21WAF1. Cancer Res. 55:4536-4539.

Chuang, L. S.-H., Ian, H.-I., Koh, T.-W., Ng, H.-H., Xu, G., Li, B.F.L. (1997): Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1. *Science* 277: 1996-2000.

Coll, J., Weeks, J., Hickey, R., Schnaper, L., Yue, W., Brodie, A., and Malkas, L.H. (1996): The human breast cell DNA synthesome: Its purification from tumor tissue and cell culture. *Oncol. Res.* 8: 435-447.

Coll, J.M., Hickey, R.J., Cronkey, E.A., Jiang, H.-Y., Schnaper, L., Lee, M.Y.W.T., Uitto, L., Syvaioja, J.E., Malkas, L.H. (1997): Mapping specific protein-protein interactions within the core component of the breast cell DNA synthesome. *Onc. Res.* 9:629-639.

Del Sal, G., Murphy, M., Ruaro, E.M., Lararevic, D., Levine, A.J., Schneider, C. (1996) Cyclin D1 and p21/waf1 are both involved in p53 growth suppression. *Oncogene* 12(1):177-185. Koeffler, H.P.(1995): Role of the Cyclin-Dependent Kinase Inhibitors in the Development of Cancer. *Blood* 86, 841-854.

Di Cunto, F., Topley, G., Calautti, Hsiao, J., Ong, L., Seth, P.K., Dotto, G.P. (1998): Inhibitory function of p21Cip1/WAF1 in differentiation of primary mouse keratinocytes independent of cell cycle control. *Science* 280:1069-1072.

Eki, T. (1994): Poly(ADP-ribose) polymerase inhibits DNA replication by human replicative DNA polymerase alpha, delta, and epsilon *in vitro*. *FEBS Letters* 336:261-266.

Fotedar, A., Cannella, D., Fitzgerald, P., Rousselle, T., Gupta, S., Doree, M., Fotedar, R. (1996): Role for cyclin A-dependent kinase in DNA replication in human S phase cell extracts. *JBC* 271(49):31627-31637.

Gadbois, D., Peterson, S., Bradbury, E.M., Lehnert, B. (1995) CDK4/cylinD/PCNA complexes during Staurosporine-induced G<sub>1</sub> arrest and G<sub>0</sub> arrest of human fibroblasts. *Exp. Cell Res.* 220:220-225.

Gartel, A.L., Serfas, M.S., Tyner, A.L. (1996) p21-Negative regulator of the cell cycle. *J. of Exp. Biol. and Med.* 213:138-149.

Goubin, F., Ducommun, B. (1995) Identification of binding domains on the p21Cip1 cyclin-dependent kinase inhibitor. *Oncogene* 10(12):2281-2287.

Gorospe, M., Holbrook, N. (1996) Role of p21 in prostaglandin A<sub>2</sub>-mediated cellular arrest and death. *Cancer Res.* 56:475-479.

Gulbis, J., Kelman, Z., Hurwitz, J., O'Donnell, M., and Kuriyan, J. (1996): Structure of the C-terminal region of p21 complexed with human PCNA. *Cell* 87:297-306

Haper, W., Adami, G., Wei, N., Keyomarsi, K., Elledge, S. (1993) The p21 cdk-interacting protein cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 75:805-816.

Hedley, D.W., Rugg, C.A., and Gelberg, R.D. (1987): Association of DNA index and S-phase fraction with prognosis of node early positive breast cancer. *Cancer Res.* 47:4729-4735.

Hindges, R., Hubscher, U. (1997): DNA polymerase  $\delta$ , an essential enzyme for DNA transactions. *Biol. Chem.* 378: 345-362.

Hirama, T., Koeffler, H.P.(1995) Role of the cyclin-dependent kinase inhibitors in the development of cancer. *Blood* 86 (3):841-854.

Hughli, Tony. (1989):Techniques in Protein Chemistry. New York, Academic Press.

Jackson, P.K., Chevalier, S., Phlippe, M., Kirschner, M.W. (1995): Early events in DNA replication require cyclin E and are blocked by p21CIP1. *J. of Cell Biol.* 130(4): 755-769.

Kallioniemi, O.-P., Hietanen, T., Mattila, J., Lehtinen, M., Lauslahti, K., and Koivula, T. (1987): Aneuploid DNA content and high S-phase fraction of tumor cells are related to poor prognosis in patients with primary breast cancer. *Europ. J. Clin. Oncol.* 23:277-283

Kallioniemi, O.-P., Blanco, G., Alavaikko, M. Hietanen, T., Matilla, J., Lauslahti, K., Lehtinen, M., and Koivula, T. (1988): Improving the prognosis value of DNA flow cytometry in breast cancer by combining DNA index and S-phase fraction. A proposed classification of DNA histograms in breast cancer. *Cancer* 62:2183-2190.

Klinton berg, S., Stal, O., Nordenskjold, B., Wallgren, A., Arvidsson, S., and Skoog, L.(1987):Proliferative index , cytosol estrogen receptor, and auxiliary node status as prognostic predictors in human mammary carcinoma. *Breast Cancer Res.Treat.* 7: 99-106

Knibleihler, M., Goubin, F., Escalas, N., Jonsson, Z., Mazarguil, H., Hubscher, U., Ducommun, B. (1996) Interaction studies between the p21Cip1/Waf1 cyclin-dependent kinase inhibitor and proliferating cell nuclear antigen (PCNA) by surface plasmon resonance. *FEBS* 391:66-70.

Kornberg, A., and Baker,T. (1992) in *DNA Replication* , 2<sup>nd</sup> edition New York, W.H. Freeman and Co.

Krishna, T., Xiang-Peng,K., Gary S., Burgers, G., Kuriyan, J. (1994): Crystal structure of the eukaryotic polymerase processivity factor PCNA. *Cell*: 79:1233-1243.

Levin D.S., Bai, W., Yao, N., O'Donnell, M., Tomkinson, A.E. (1997): An interaction between DNA ligase I and proliferating cell nuclear antigen: Implications for Okazaki fragment synthesis and joining. *P.N.A.S.* 94: 12863-12868.

Li, J.J., Kelly T.J. (1984) Simian virus 40 DNA replication *in vitro* *P.N.A.S.* 81:6973-6977.

Li, R., Waga, S., Hannon, G., Beach, D., Stillman, B. (1994) Differential effects by the p21 CDK inhibitor on PCNA-dependent replication and repair. *Nature* 371:534-537.

Li, R., Hannon, G.J., Beach, D., Stillman, B. (1996): Subcellular distribution of p21 and PCNA in normal and repair-deficient cells following DNA damage. *Current Bio.* 6 (2):189-199.

Lin, S., Hickey, R.J., Malkas, L.H. (1997): The isolation of a DNA synthesome from human leukemia cells. *Leuk. Res.* 21 (6):501-512.

Lin, S., Hickey, R.J., Malkas, L.H. (1997): The biochemical status of the DNA synthesome can distinguish between permanent and temporary growth arrest. *Cell Growth and Diff.* 8:1359-1369.

Loor, G., Zhang, S.-J., Zhang, P., Toomey, N.L., Lee, M.Y.W.T. (1997): Identification of DNA replication and cell cycle proteins that interact with PCNA. *Nuc. Ac. Res.* 25(24): 5041-5046.

Luo, Y., Hurwitz, J., Massague, J. (1995) Cell cycle inhibition by independent CDK and PCNA binding domains in p21Cip1. *Nature* 375:159-161.

Malapetsa, A., Noe, A.J., Poirier, G.G., Desnoyers, S., Berger, N.A., Panasci, L.C. (1996): Identification of a 116 kDa protein able to bind 1,3-bis(2-chlorethyl)-1-nitrosourea-damaged DNA as poly(ADP-ribose) polymerase. *Mut. Res.* 363:41-50.

Malkas, L.H., Hickey, R. J., Li, C.-J., Pederson, N., and Baril, E.F. (1990): A 21S enzyme complex from HeLa cells that functions in simian virus 40 DNA replication *in vitro*. *Biochemistry* 29:6362-6374.

McDonald, R., Wu, G., Waldman, T., El-Deiry, S. (1996) Repair defect in p21WAF1/CIP1-/- human cancer cells. *Cancer Res.* 56:2250-2255.

Meyer, J.S., Prey, M.U., Babcock, D.S. and McDivitt, R.W. (1986): Breast carcinoma, cell kinetics, morphology, stage and host characteristics. A thymidine lab study. *Lab Invest.* 54:41-51.

Mozzherin, D.J., McConnell, M., Jasko, M.V., Krayevsky, A.A., Tan, C.-K., Downey, K.M., Fischer, P.A. (1996); Proliferating cell nuclear antigen promotes misincorporations catalyzed by calf thymus DNA polymerase  $\delta$ . *271(49): 31711-31717.*

Mozzherin, D.J., Shibutani, S., Tan, C-K., Downey, K.M., Fischer, P.A. (1997): proliferating cell nuclear antigen promotes DNA synthesis past template lesions by mammalian DNA polymerase  $\delta$ . P.N.A.S. 94: 6126-6131.

Nakanishi, M., Robetorye, R., Pereire-Smith, O., Smith, J. (1995) The C-terminal region of p21SDI1/Waf1/Cip1 is involved in proliferating cell nuclear antigen binding but does not appear to be required for growth inhibition. J. of Biol. Chem. 270(29):17060-17063.

Nakanishi, M., Adami, G., Robetorye, R., Noda, A., Venable, S., Dimitrov, D., Pereira-Smith, O., Smith, J. (1995) Exit from G0 and entry into the cell cycle of cells expressing p21SDI1 antisense RNA. P.N.A.S. 92:4352-4356.

Nie, J., Sakamoto, S., Song, D., Qu, Z., Ota, K., Taniguchi, T. (1998): Interactions of Oct-1 and automodification domain of poly(ADP-ribose) synthetase. FEBS Letters 424:27-32.

Pan, Z-Q., Chen, M., Hurwitz, J. (1993): The subunits of activator 1 (replication factor C) carry out multiple functions essential for proliferating-cell nuclear antigen-dependent DNA synthesis. P.N.A.S. 90:6-10.

Podust, V., Podust, L., Goubin F., Ducommun, B. and Hubscher, U. (1995): Mechanisms of inhibition of proliferating cell nuclear antigen-dependent synthesis by the cyclin-dependent kinase inhibitor p21. Biochemistry 34:8869-8875.

Sato, T., Akiyama, F., Sakamoto, G., Kasumi, F., and Nakamura, Y. (1991): Accumulation of genetic alterations and progression of primary breast cancer. Cancer Res. 51:5794-5799.

Smith, M.L., Chen, I-T., Zhan, Q., Bae, I., Chen, C-Y., Gilmer, T.M., Kastan, M.B., O'Connor, P.M., Fornace, A.J., Jr. (1994): Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science 266: 1376-1379.

Stillman, B. (1996) Cell cycle control of DNA replication. Science 274:1659-1664.

Tan, C-K., Castillo, C., So, A., Downy, K. (1986): An auxiliary protein for DNA polymerase  $\delta$  from fetal calf thymus. J. Biol. Chem. 261:12310-12316.

Tom, T.D., Malkas, L.H., Hickey, R.J. (1996): Identification of multiprotein complexes containing DNA replication factors by native immunoblotting of HeLa cell protein preparations with T-antigen-dependent SV40 DNA replication activity. J. of Cell. Biochem. 63:259-267.

Tournier, S., Leroy, D., Goubin, F., Ducommun, B., Hyams, J. (1996) Heterologous expression of the human cyclin-dependent kinase inhibitor p21Cip1 in the fission yeast

*Schizosaccharomyces pombe* reveals a role for PCNA in the chk1+ cell cycle checkpoint pathway. Mol. Cell Biol. 7:651-662.

Towbin, H. Strachelin, and Gordon, J. (1979): Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. P.N.A.S. 76:4350-4355.

Ueda, K. and Hayaishi, O. (1985) ADP-Ribosylation. Ann. Rev. Biochem. 54:73-100.  
Gretch D.R., et al. (1987) Anal. Biochem. 163:270.

Umar, A., Buermeyer, A., Simon, J., Thomas, D., Clark, A., Liskay, R., Kunkel, T. Requirement for PCNA in DNA mismatch repair at a step preceding DNA resynthesis. (1996) Cell 87:65-73.

Waga, S., Hannon, G., Beach, D., Stillman, B. (1994) The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature 369:574-578.

Wang, Z.-Q., Auer, B., Stingl, L., Berghammer, H., Haidacher, D., Schweiger, M., Wagner, E.F. (1995): Mice lacking ADPRT and poly(ADP-ribosyl)ation develop normally but are susceptible to skin disease. Genes and Dev. 9:509-520.

Warbrick, E., Lane, D.P., Glover, D.M., Cox, L.S. (1997): Homologous regions of Fen1 and p21Cip1 compete for binding to the same site on PCNA: a potential mechanism to co-ordinate DNA replication and repair. Oncogene 14: 2313-2321.

Warbrick, E. (1998): PCNA binding through a conserved motif. BioEssays 20:195-199.

Wu, Y., Hickey, R.J., Lawlor, K., Wills, P., Yu, F., Ozer, H., Starr, R., Yan-Quan, J., Lee, M. and Malkas, L.H. (1994): A 17S form of DNA polymerase from mouse cells mediates the *in vitro* replication of polyomavirus DNA. J. of Biochem. 54:32-46.

Xiong, Y., Zhang, H., Beach, D. (1992) D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell 71:505-514.

Yan G., Ziff, E.B. (1995): NFG regulates the PC12 cell cycle machinery through specific inhibition of the Cdk kinases and induction of cyclin D1. J. of Neurosc. 15(9): 6200-6212.

Yan, H., Newport, J. (1995) An analysis of the regulation of DNA synthesis by cdk2, Cip1, and licensing factor. J. Cell. Biol. 129(1):1-15.

Zhang, P., Sun, Y., Hsu, H., Zhang, L., Zhang, Y., Lee, M.Y.W.T. (1998) The interdomain connector loop of human PCNA is involved in a direct interaction with human polymerase  $\delta$ . JBC 273(2): 713-719.